Yersinia enterocolitica prevalence, on fresh pork, poultry and beef meat at retail level, in France.

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Abstract
Y. enterocolitica is a zoonotic agent, and the third bacterial cause of human entiritis in Europe. The objective of this study was to assess consumer exposure to the pathogen *Y. enterocolitica* through meat consumption over a one-year period, in France. In this context, the prevalence of *Y. enterocolitica* was established on samples of fresh pork, beef and poultry collected at retail level in France. Of the 649 samples, 5.1% (34) were positive for *Y. enterocolitica*. No significant difference in prevalence between the categories of fresh meat was observed: the prevalence was 5.2 % for pork, 5.2% for beef and 5.9% for poultry meat. However, tongues of pork were highly contaminated by *Y. enterocolitica* (12.5%) compared to other type of meat.

Although the isolation methods of *Y. enterocolitica* was done by a method promoting the detection of pathogenic biotypes 1B, 2, 3, 4 and 5 (enrichment ITC and streaking on CIN), only strains carrying biotype 1A were isolated. Strains of biotype 1A are considered non-pathogenic for humans. However, recent studies indicate that biotype 1A strains isolated from clinical cases have in their genomes genes known to play a role during disease. The presence of the three virulent genes *inv, myfA* and *ystB* were tested; the gene *ystB* being strongly related to the clinical biotype 1A. The three genes were detected for some of our strains and 71% of our isolates carried the *ystB* gene. Thus, these strains can be potentially pathogenic for humans.

Introduction

Y. enterocolitica is an important food-borne enteropathogen, known to cause a wide variety of clinical symptoms ranging from mild gastroenteritis to invasive syndromes like terminal ileitis (Bottone, 1999). *Y. enterocolitica* is the third bacterial cause of human enteritis in Europe (EFSA and ecdc, 2012).

This pathogen is of particular concern for consumers' safety because it is able to growth in food stored at refrigeration temperatures without apparent signs of spoilage.

The species *Y. enterocolitica* is divided into six biotypes. The biotype 1A generally regarded as nonpathogenic and the pathogenic biotypes 1B, 2, 3, 4, 5 (Wauters *et al,* 1988). In France and most other countries worldwide, biotype 4 is the most prevalent biotype isolated from humans (69%), followed by biotype 2 (30%) and biotype 3 (Savin and Carniel, 2008). Genes involved in virulence have been characterized. Among them, the gene *inv* for invasive gene mediates cell invasion, the *myf*-gene encodes the production of fibrillae Myf and the *yst*-gene which encodes enterotoxin.

Y. enterocolitica has frequently been isolated from animals, food and environment (Falcao *et al*., 2006). Pigs are considered the principal reservoir for human pathogenic strain of *Y. enterocolitica*. Pigs do not develop clinical signs, but they do carry *Y. enterocolitica* in their oral cavity, on tongues and tonsils, and in lymph nodes, and excrete this bacterium in their feces (Thibodeau et al., 1999).

It is assumed that the main sources of infection in humans are pig and pork products (Bottone, 1999; Gousia *et al*., 2011), however *Y. enterocolitica* have been found on other type of meat (Bonardi *et al*., 2010).

A one year survey, done on pig tonsils at slaughterhouse in 2010-2011 by our laboratory, estimated that 13.7% of the pigs carried *Y. enterocolitica* and that 74.3% of the pig batches contained at least one positive pig (Fondrevez, 2012). Most of the strains were of biotype 4. In this context we investigate the occurrence of *Y. enterocolitica* in the major meat species, pork, beef and poultry at retail level in France. The isolated stains were then screened for the presence of virulence genes.

Material and Methods

Food samples

A total of 649 raw meat samples was collected at retail level in France during the year 2012. Samples were consisted in raw meat of pork (n=237), beef (n=210) and chicken (n= 202). All the samples were kept at 4°C during transport and during

storage before analysis. The analysis was done within 4 days after purchase.

Microbiological analysis

The presence of *Y. enterocolitica* in meat samples was assessed as follow: 25g of meat were diluted 1:10 in peptone salt broth (AES chemunex) and homogenized in stomacher for 90s. One ml of this suspension was then placed in a tube containing 9 ml irgasan–ticarcillin–potassium chlorate (ITC) broth (Bio-Rad, Marnes La Coquette, France).

The ITC enrichment broth was incubated for 48h at 25°C. Streaking was done on cefsulodin–irgasan–novobiocin (CIN) agar plates (*Yersinia* Selective Agar Base and *Yersinia* Selective Supplement, Oxoid, Basingstoke, UK).

After 24h at 30°C, we checked for the presence of typical colonies on CIN plates. We then streaked a maximum of four characteristic colonies per sample on plates containing the *Y. enterocolitica* chromogenic medium (YeCM) (prepared in the laboratory as described by Weagant, 2008), for the presumptive selection of *Y. enterocolitica* isolates carrying pathogenic biotypes (red "bull's-eye" colonies) and of *Y. enterocolitica* isolates carrying the biotype 1A (bleu-purple colonies). Each isolate was then subcultured on Plate Count Agar (PCA) plates (AES, Bruz, France) and incubated at 30°C for 24h for biochemical assays. The ability of isolate to degrade urea was used to confirm that the isolates belong to *Yersinia*. Strains were stored in peptone glycerol broth, at −80°C.

DNA extraction and Real-Time PCR for detection of virulence genes

Real Time PCR was used to evaluate the presence of virulence genes *inv*, *myfA* and *ystB*. Strains were sub-cultured on PCA at 30°C for 24h. DNA was extracted from some colonies with QIAamp DNA mini kit (Qiagen, USA) following the manufacturer's instructions. The PCRs were performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California), in a final volume of 25µl with the Sybr® Green JumpstartTM Taq ReadyMix TM (Sigma-Aldrich, Saint Louis, Missouri) at 1X. The genes *inv* (Rasmussen *et al*., 1994), *myfA* (Kot and Trafny, 2004) and *ystB* were detected with specific primers as indicated in table 1. The final concentration of primers in the PCR reaction was 0.3 µM. The amplification conditions for each gene are detailed in Table 1.

Table 1: Primer sequences and PCR conditions for detection of virulence genes by Real-Time PCR

Genes	Primer sequence	First step	Cycle of amplification	Melt Curve	Size in bp	Expected Tm
inv	F-CTG TGG GGA GAG TGG GGA AGT TTG G R-GAA CTG CTT GAA TCC CTG AAA ACC G	94°C 2 min	34 cycles of Denaturation 94°C 60 sec Annealing 61°C 60 sec Extension 72°C 30 sec	56°C -> 95°C, increment of 0.5°C for 5 sec	570	87.5°C
myfA	F - CAG ATA CAC CTG CCT TCC ATC T R- CTC GAC ATA TTC CTC AAC ACG C	94°C 2 min	35 cycles of Denaturation 94°C 60 sec Annealing 58°C 90 sec Extension 72°C 90 sec	56°C -> 95°C, increment of 0.5°C for 5 sec	272	84.5°C
ystB	F-AAA GCG TGC GAT ACT CAG AC R-CAG CAT ACC TCA CAA CAC CA	95°C 5 min	34 cycles of Denaturation 94°C 30 sec Annealing 55°C 30 sec Extension 72°C 30 sec	56°C -> 95°C, increment of 0.5°C for 5 sec	68	79°C

Results

A total of 649 samples, including raw meat of pork, beef and chicken were obtained from different supermarkets in France. Of the 649 samples, 5.1% (32) were positive for *Y. enterocolitica*. Although the isolation methods of *Y. enterocolitica* was done by a method promoting the detection of pathogenic biotypes 1B, 2, 3, 4 and 5 (enrichment ITC and streaking on CIN), only strains carrying biotype 1A were isolated. The prevalence of this biotype in the different categories of meat was 5.2% for pork, 5.2% for beef and 5.9% for poultry meat (Table 2).

No significant difference in prevalence of *Y. enterocolitica* was observed between the three categories of fresh meat. Among pork meat sample, the tongues showed the highest prevalence (12.5%) followed by the minced meat (6.9%) (Table 3).The other pork meats like pork chop, fillet and roast were very little contaminated (2.1%). The occurrence of *Y. enterocolitica* in tongue was significantly higher than that of other pork meats (χ^2; p=0.05) but not of that of minced meat (χ^2; p=0.42).

Because recent studies indicate that biotype 1A strains may have in their genomes genes known to play a role during disease, the strain were screened for the presence of virulence genes *inv, myfA* and *ystB*. The incidence of virulence genes detected in the three categories of meat is presented in table 4.

Among the strains tested, only six lacked the virulence genes tested. The *inv*, *myfA and ystB* genes were detected with an incidence of 65%, 12% and 71%, respectively. The *inv*, and *ystB* virulent genes were detected in the various categories of meat. Only two isolates from pork and two isolates from chicken carried the gene m*yfA*. This gene is not detected in isolates obtained from beef meat.

Table 2 : *Y. enterocolitica* contamination rate of the three categories of meat

Categories of meat	*Number of samples*	*Number of positive samples*	*% of positive samples*
Pork	237	11	5.2
Beef	210	11	5.2
Poultry	202	12	5.9
Total	649	34	5.1

Table 3 : *Y. enterocolitica* contamination rate of the three types of pork meat

types of pork meat	*Number of samples*	*Number of positive samples*	*% of positive samples*
Tongue	24	3	12.5
Minced meat	72	5	6.9
Other pork meats	141	3	2.1
Total	237	11	5.2

Table 4: Distribution of virulence genes among the categories of meats

Categories of meat	n° of strains	Virulence genes					
		inv		*myfA*		*ystB*	
		number	%	number	%	number	%
Pork	11	6	55	2	18	7	64
Beef	11	8	72	0	0	10	91
Poultry	12	8	67	2	17	7	58
Total	34	22	65	4	12	24	71

Discussion

In the present work, *Y. enterocolitica* were detected in the three main category of meat. The prevalence was about 5% to 6% for raw pork, poultry and beef meat. This prevalence was lower than that observed in Italy by Bonardi *et al.* (2010) which detected a contamination rate of 15.2% for raw pork meat and 32.5% for chicken meat. In our study, tongues of pork were highly contaminated by *Y. enterocolitica* (12.5%) compared to other type of meat. This is coherent with the carriage of *Y. enterocolitica* by pig for which *Y. enterocolitica* is particularly present in their oral cavity (Thibodeau et al., 1999). The great majority of *Y. enterocolitica* isolates from food product belongs to biotype 1A (Bonardi *et al.*, 2010), which is in agreement with our results. Indeed, none *Y. enterocolitia* carrying enteropathogenic biotype were detected in the present study although the isolation methods used promote the detection of pathogenic biotypes. Like previous studies (Bonardi *et al.*, 2010; Falcao *et al.*, 2006; Grant *et al.*, 1998), the predominant genotype for virulent genes was *inv*+ and *ystB*+. The presence of *ystB* is strongly related to the clinical biotype 1A (Grant *et al.*, 1998) and is found in 71% of our isolates. The presence of the three genes *ystB*, *inv* and *myfA* for some of our strains reveals that these strains can be potentially pathogenic for humans.

Conclusion

Our study indicates that consumption of meat from pork, beef or poultry presents a low risk of *Y. enterocolitia* for humans. Indeed, the prevalence of *Y. enterocolitia* is low and strains carried the biotype 1A considered as non-pathogenic for humans. However, some of these strains have several genes associated with pathogenicity especially the *ystB* gene often associated with clinical cases with this biotype. This risk should not be ignored.

Acknowledgements

The authors gratefully acknowledge Aurore Fablet for technical help.

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